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B-*raf* Oncogene: Activation by Rearrangements and Assignment to Human Chromosome 7

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cDNA clones presumably containing a transforming gene were isolated from transformed NIH3T3 cells, which were obtained by the transfection of DNA from peripheral blood lymphocytes of a familial adenomatous polyposis patient. Sequence analysis of the cDNA clones showed that the gene contained an activated B-*raf*, the 5′ half of which was replaced by the human small nuclear ribonucleoprotein E protein gene and an unknown gene. A partial genomic physical map of the transforming gene was made on the basis of the physical map of the cDNA clones, indicating that rearrangements had occurred during the transfection. The human B-*raf* gene was shown to be located on chromosome 7 by Southern blotting analysis of rodent-human somatic cell hybrid DNA using the B-*raf* cDNA as a probe.

Key words: Oncogene — B-*raf* — DNA transfection — Chromosomal localization

The v-*raf* oncogene was originally isolated from a murine transforming retrovirus, 3611-MSV,[1] and is a *gag*-fused truncated version of mouse c-*raf*. Southern blot analysis with v-*raf* cDNA probes identified two *raf* genes, c-*raf*-1 and A-*raf*, in humans.[2,3] A third member of the family, B-*raf*, was isolated by DNA transfection.[4] The three *raf* proteins show the greatest stretch of homology in the carboxy-terminal half,[3–5] which has been shown to exhibit serine- and threonine-specific protein kinase activity.[6,7] Physiologically, the *raf* protein is supposed to transduce an intracellular signal from the cytoplasmic membrane to the nucleus.[8–12]

Transforming *raf* genes have frequently been obtained by transfection of genomic human and rodent DNAs into NIH3T3 cells. Activated forms of the *raf* protein were found to lack the amino-terminal sequence and to produce fusion proteins with the products of other genes.[13–17] Several studies have shown that the mechanism underlying oncogenic activation of these transforming versions comprises alteration of the putative regulatory domain of the amino-terminal half of the *raf* gene.[18–22]

Although c-*raf*-1 has been well studied, B-*raf* has only been mentioned in two reports[4,5] and thus has not been analyzed sufficiently yet. During our studies on the transforming activities of DNAs from peripheral blood lymphocytes (PBL) of familial adenomatous polyposis (FAP) patients,[23] we found an activated B-*raf* oncogene. We report here the characterization of the transforming gene.

MATERIALS AND METHODS

Cells and culture NIH3T3 cells and the transformants (S378A) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% or 5% calf serum under a humidified atmosphere of 5% CO_2 in air at 37°C. S378A cells were the primary transformants induced by a PBL DNA sample from a FAP patient (case G1III6).[23]

DNA and RNA blotting analysis DNA samples were transferred from agarose gels to nitrocellulose filters as described by Southern.[24] Poly(A)$^+$ RNA samples were resolved on formaldehyde-agarose gels and transferred to nitrocellulose filters by standard procedures. Hybridization and washes were carried out by the method described previously.[25]

cDNA library construction Total RNA was isolated from S378A cells, using the CsCl/guanidium isothiocyanate method.[26] Poly (A)$^+$ RNA was obtained by passing the total RNA through an oligo(dT)-cellulose column and a cDNA library was constructed from the poly(A)$^+$ RNA using the bacteriophage vector, λgt10.[27] Approximately 1.2×10^6 recombinant phages were plated onto *Escherichia coli* strain C600hfl$^-$. Phage DNA was transferred to duplicate nitrocellulose filters and denatured. Duplicate filters were hybridized with a ^{32}P-labeled 1.0 kb *Hin*dIII-*Hin*dIII fragment of the genomic EMBL 3 clone, λHTB3-1 (Fig. 4, 3B), at 42°C for 16 h in a hybridization solution. The filters were washed in a buffer containing 0.1×SSC and 0.1% SDS at 50°C. Plaques with positive signals were purified by means of successive plaque-hybridization. Lambda phage DNA was purified for further analysis as described previously.[28]

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Restriction mapping Restriction mapping of the cDNA and genomic clones was performed by standard techniques using single and double digests of phage or genomic DNA as previously described.[29]

DNA sequencing A cDNA subfragment was inserted into pUC118. Nucleotide sequence analysis of cDNA was carried out by the chain termination method[30] using a deaza-sequencing kit (Takara Shuzo Co., Kyoto) on double-stranded plasmid DNA. Each subclone was sequenced in both directions.

Computer analysis A homology search among known DNA and protein sequences was performed using a previously developed algorithm[31] against the GenBank nucleic acid database and the National Biomedical Research Foundation protein data base.

RESULTS

cDNA cloning We obtained NIH3T3 transformants by means of a tumorigenicity assay after DNA transfection of PBL of a FAP patient (case G1III6). The transforming gene was partially cloned using the EMBL 3 phage as a vector.[23] A 1.0 kb *Hin*dIII-*Hin*dIII DNA fragment, 3B, of a clone, λHTB3-1 (Fig. 4), was shown to contain evolutionally well-conserved sequences, as judged on cross-species hybridization (data not shown). Using this fragment as a probe, transcripts of 2.3 kb and 1.8 kb in length were detected in poly(A)$^+$ RNA from the G1III6 PBL-derived tumors. The 3B fragment was used to screen the cDNA library.

A cDNA library was constructed from poly (A)$^+$ RNA of the first cycle tumor-derived cells, S378A. The screening of 1.2×10^6 plaques with this probe resulted in the isolation of two clones, designated as λHT 11 and 18. Restriction enzyme mapping demonstrated that the two clones overlapped each other (Fig. 1). In order to obtain full-length cDNAs of the 1.8 kb and 2.3 kb transcripts, the same cDNA library was subsequently screened with a 0.2 kb *Eco*RI-*Bam*HI subfragment of λHT 11 (Fig. 1, probe C) and a 0.4 kb *Hae*III-*Eco*RI subfragment of λHT 18 (Fig. 1, probe D). The overlapping cDNA clones, λHT 11-2, 11-4, 11-6 and 18-7, were isolated. A composite restriction map for the six overlapping clones spanned a total length of 1.5 kb.

Blot hybridization analysis of poly(A)$^+$ RNA from S378A cells showed that probe C (Fig. 1) hybridized strongly to the 2.3 kb and 1.8 kb RNAs (Fig. 2), as described previously.[23] When the same probe was used, Southern blot hybridization analysis of DNA samples from the third cycle cells, S 424 and S 448, showed strongly hybridized bands of human origin in addition to the weakly hybridized mouse-type band (data not shown). Therefore, it is most likely that the cloned cDNA sequences comprise the transforming gene of the G1III6 sample.

cDNA sequence The nucleotide sequence of the cDNA was determined. The 3′ end of the cDNA did not show a poly(A)$^+$ RNA sequence. Since there is only one consensus translation initiation sequence[32] in the cDNA sequence, the predicted total open reading frame comprised 1194 nucleotides or 398 amino acids. A homology search of the entire cDNA sequence was performed with the GenBank nucleic acid database, which revealed that the cDNA consists of three regions. The nucleotide sequence of the 5′-terminal 342 bp of the cDNA clone, corresponding to fragments A and B (Fig. 1), gave the

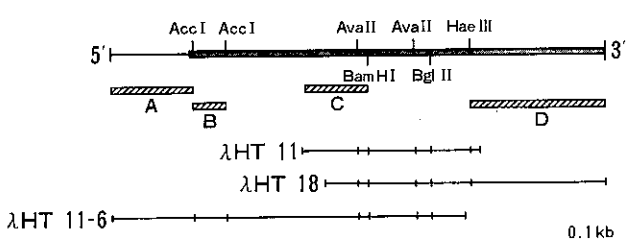

Fig. 1. cDNA clones representing the transforming gene and the composite restriction endonuclease map. The region derived from human B-*raf* is indicated by a stippled bar. The hatched bars indicate the fragments which were used as probes: A, 0.25 kb *Eco*RI-*Acc*I fragment; B, 0.1 kb *Acc*I-*Acc*I fragment; C, 0.2 kb *Eco*RI-*Bam*HI fragment; and D, 0.4 kb *Hae*III-*Eco*RI fragment.

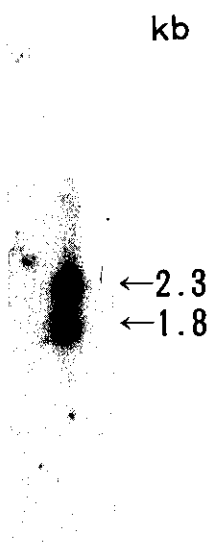

Fig. 2. Expression of the activated B-*raf* gene in the transformant. A sample (2 μg) of poly(A)$^+$ RNA from S378A cells was analyzed by blot hybridization using a 0.2 kb *Eco*RI-*Bam*HI fragment of the cDNA clone, λHT-11, as a probe.

predicted amino acid sequences illustrated in Fig. 3. The sequence downstream of position 241 exactly corresponded to the kinase domain of the reported human B-*raf* gene.[4,5] No mutation was found in the coding sequence of B-*raf*, suggesting that the activation of B-*raf* was due merely to a DNA rearrangement. The sequence upstream of position 174 was identical to exons 1–3 of the human small nuclear ribonucleoprotein (snRNP) E protein gene.[33] However, as to the nucleotide sequence from position 175 to 240 and the predicted amino acid sequence, significant homology could not be found in either the GenBank nucleic acid database or the National Biomedical Research Foundation protein data base.

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                                                Met Ala Tyr Arg Gly Gln
GTG CGG GTG TGC TCT TTG TGA AAT TCC ACC ATG GCG TAC CGT GGC CAG    48

Gly Gln Lys Val Gln Lys Val Met Val Gln Pro Ile Asn Leu Ile Phe
GGT CAG AAA GTG CAG AAG GTT ATG GTG CAG CCC ATC AAC CTC ATC TTC    96

Arg Tyr Leu Gln Asn Arg Ser Arg Ile Gln Val Trp Leu Tyr Glu Gln
AGA TAC TTA CAA AAT AGA TCG CGG ATT CAG GTG TGG CTC TAT GAG CAA   144

Val Asn Met Arg Ile Glu Gly Cys Ile Ile Asn Asp Gln Ser Met Gly
GTG AAT ATG CGG ATA GAA GGC TGT ATC ATT AAT GAC CAG TCC ATG GGT   192
                                    ↑
Glu Met Gln Ile Ile Gly Gly Asp Asp Leu Ser Thr Leu Ala Gly Lys
GAG ATG CAG ATA ATC GGA GGC GAT GAT CTT TCA ACG CTG GCT GGA AAG   240
                                                              ↑
Lys Thr Leu Gly Arg Arg Asp Ser Ser Asp Asp Trp Glu Ile Pro Asp
AAA ACA CTT GGT AGA CGG GAC TCG AGT GAT GAT TGG GAG ATT CCT GAT   288

Gly Gln Ile Thr Val Gly Gln Arg Ile Gly Ser Gly Ser Phe Gly Thr
GGG CAG ATT ACA GTG GGA CAA AGA ATT GGA TCT GGA TCA TTT GGA ACA   336

Val Tyr
GTC TAC
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Fig. 3. The nucleotide sequence of the 5′-terminal 342 bp of the cDNA clones and the deduced amino acid sequence in-frame with that of B-*raf*. The arrows indicate the positions of recombination points. The solid bar indicates the putative ATP-binding site.

Structure of the activated B-*raf* gene Seven subfragments of the cDNA clones were used to probe Southern blots containing different digests (*Eco*RI, *Hin*dIII and *Sal*I) of the genomic phage clones[23] and normal human genomic DNA. As shown in Fig. 4, the cDNA clones hybridized over a length of 70 kb of genomic DNA. The 1.0 kb *Hin*dIII-*Hin*dIII fragment (3B) of the genomic clone, λHTB 3-1, which was used to screen the cDNA library, was sequenced. This fragment contained 135 nucleotides of the cDNA sequence and splice acceptor and splice donor consensus sequences at the intron boundaries (data not shown). This exon corresponds to exon 16 of c-*raf*-1.[34]

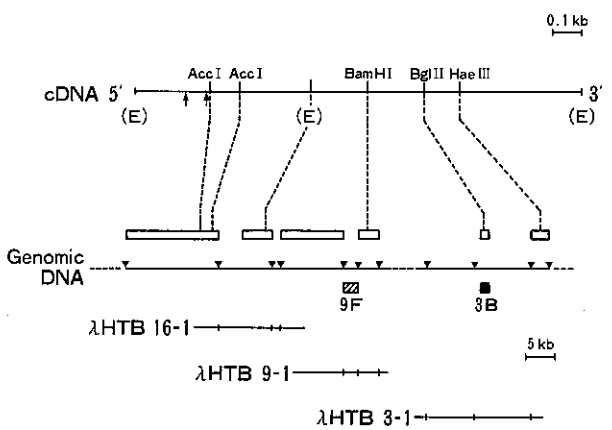

Fig. 4. Genomic restriction map of the transforming gene derived from EMBL 3 phage clones. Open bars indicate genomic fragments that hybridized to cDNA sequences and are delimited by restriction sites for *Eco*RI (▼), *Sal*I and *Hin*dIII (not shown in the genomic restriction map). The top line represents the cDNA map. The arrows indicate the recombination points. The positions of a number of "key" restriction sites (*Acc*I, *Bam*HI, *Bgl*II, *Hae*III and *Eco*RI [(E), derived from linkers]) in the cDNA are indicated on the open bars by dashed lines. The solid box represents the 1.0 kb *Hin*dIII-*Hin*dIII fragment (3B) of the λHTB 3-1 clone which was used as the probe for the screening. The hatched box represents the 2.0 kb *Eco*RI-*Eco*RI fragment (9F) of the λHTB 9-1 clone which was used as the probe for hybridization with rodent-human hybrid cell DNAs to determine the chromosomal localization.[23]

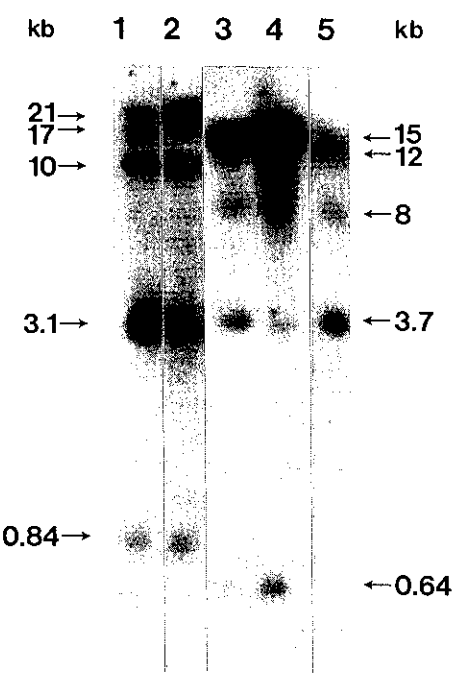

Fig. 5. Occurrence of recombination in primary transformant DNA but not in the original peripheral blood lymphocyte DNA. Each DNA was digested with *Hin*dIII and then probed with the 0.25 kb *Eco*RI-*Acc*I cDNA fragment (Fig. 1, probe A). Lane 1, PBL DNA; lane 2, human normal genomic DNA (TIG 7 cells); lane 3, S378A DNA; lane 4, S402 DNA; and lane 5, NIH3T3 DNA.

Recombination occurred during transfection When the 0.25 kb *Eco*RI-*Acc*I fragment of cDNA (Fig. 1, probe A), which consisted of the snRNP E protein gene, an unknown gene and only 12 bp of B-*raf*, was used as a probe, a *Hin*dIII digest of human genomic DNA gave the 21 kb, 17 kb, 10 kb, 3.1 kb and 0.84 kb bands in Fig. 5. It is most likely that the 10 kb band was derived from the snRNP E protein gene[33] and other bands from an unknown gene. DNAs from S378A and the second cycle cells, S402, gave 12 kb, 8 kb and 3.7 kb bands derived from NIH3T3 cell DNA, and amplified 15 kb and 0.64 kb bands from the fused gene (Fig. 5, lanes 3 and 4). These recombinant fragments were not detected in the original lymphocyte DNA from the patient. Therefore, recombination would appear to have occurred during the transfection process.

Chromosomal localization of human B-*raf* gene To determine the human chromosomal location of the B-*raf* gene, we performed Southern blotting analysis of DNA from 10 different rodent-human somatic cell hybrids

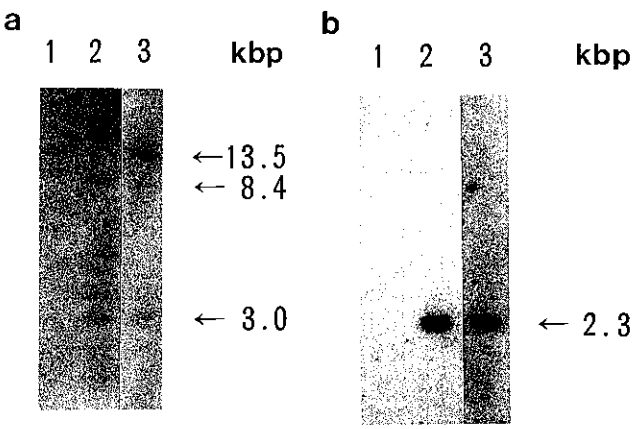

Fig. 6. (a) Chromosomal localization of B-*raf*. *Eco*RI-digested DNA from mouse (lane 1), human (lane 2) and rodent-human hybrid cell clone, 7D3 (lane 3) was hybridized with the 0.2 kb *Eco*RI-*Bam*HI fragment of the B-*raf* cDNA (Fig. 1, C). (b) Hybridization of the same DNAs as in (a) with the *met* probe.

Table I. Detection of the Human B-*raf* Gene in Rodent-Human Hybrid Cells

Human chromosome	Hybrid clone[a]									
	2-1	7-1	1-6	3-2	3-5	7D3	1B1-36	4C2	2C3	3B5
1	+	−	−	−	(+)	−	−	−	−	−
2	−	[+]	−	−	−	−	−	−	+	+
3	−	+	+	−	−	−	−	−	−	−
4	−	−	+	−	(+)	+	−	+	+	(+)
5	+	−	[+]	−	+	−	−	+	+	[+]
6	+	−	−	−	[+]	−	−	−	+	+
7	−	−	+	+	+	+	+	+	+	+
8	−	−	−	−	+	+	−	−	+	+
9	−	−	−	−	−	−	−	−	−	−
10	+	−	−	−	−	−	−	−	+	+
11	+	−	(+)	−	+	+	−	−	−	−
12	+	+	(+)	−	+	[+]	−	−	−	+
13	+	+	−	+	+	+	+	−	−	+
14	−	+	−	+	+	+	−	+	+	[+]
15	−	−	(+)	−	+	[+]	+	−	[+]	−
16	+	−	−	−	+	−	−	−	+	−
17	+	+	+	+	+	+	+	+	−	−
18	−	−	+	+	+	+	−	−	−	+
19	+	−	[+]	−	+	+	+	−	[+]	+
20	−	−	−	−	+	+	[+]	+	+	+
21	+	+	−	−	+	+	[+]	+	+	+
22	−	−	−	−	+	−	−	−	+	+
X	+	−	+	−	−	[+]	−	+	+	+
Y	−	−	−	−	−	−	−	−	−	−
B-*raf* cDNA[b]	N	N	P	P	P	P	P	P	P	P
Human *met* gene	N	N	P	P	P	P	P	P	P	P

a) [+]=10–19%, (+)=20–29%, +=>30%.
b) P: positive, N: negative.

containing unique complements of the human chromosome, using a 0.2 kb *Eco*RI-*Bam*HI fragment of the B-*raf* cDNA (Fig. 1, C). An example of results of such hybridization studies is shown in Fig. 6a. The B-*raf* probe detected two human *Eco*RI fragments which are present in the human DNA (lane 2) and the hybrid DNA (lane 3). A mouse B-*raf*-specific *Eco*RI fragment was detected in mouse DNA (lane 1) and in hybrid DNA (lane 3). The results are most consistent with assignment of the B-*raf* gene to chromosome 7 (Table I). To confirm these initial results, we hybridized the same DNAs to the *met* probe, which is known to be located on chromosome 7 (Fig. 6b). The 1.6 kb human *met* probe[35] showed the same hybridizing pattern as the B-*raf* probe (Table I). These data clearly show that the human B-*raf* gene is located on chromosome 7.

DISCUSSION

We reported here the molecular cloning of cDNA and the genomic structure of the activated B-*raf* found in the primary transformant. Nucleotide sequence analysis of the activated B-*raf* cDNA revealed that the mRNA contained sequences derived from the human snRNP E protein gene, the kinase domain of the human B-*raf* gene and an unknown gene. The fused gene was considered to code for a fusion protein consisting of the 48 amino-terminal amino acid residues of the snRNP E protein encoded by exons 1–3, 22 amino acid residues of an unknown protein and the 328 carboxy-terminal amino acid residues of the B-*raf* protein, which contained the kinase domain including the putative ATP-binding site required for catalytic activity.

Since there is no consensus translation initiation site in the activated B-*raf* sequence, the initiation complex of the snRNP E protein gene seems to act as that of the activated B-*raf*. It is therefore speculated that the activated B-*raf* in our case is controlled by the promoter of the snRNP E protein gene.

The recombination occurred at the position 69 bp upstream of the putative ATP-binding site. The recombination point in another case[4] is approximately 174 bp upstream of that in our case. In both cases, a cysteine finger domain (divalent cation- or DNA-binding site, or both) and a serine- and threonine-rich domain (phosphorylation site) were lost, and a kinase domain retained. As these three domains are highly conserved among *raf* family members, these domains have been called CR1, CR2 and CR3, respectively.[5,21] According to the results of studies on c-*raf*, oncogenic activation of *raf* kinases can be achieved by removal of CR1 or CR2, or by means of a linker insertion into CR2, but it requires the retention of an active kinase domain including the putative ATP-binding site.[21] In addition, A-*raf* became tumorigenic when its carboxy-terminal half was fused with viral *gag* and expressed using a retroviral vector. Thus, the mechanism underlying B-*raf* activation also seems to be related to alteration or deletion of N-terminal amino acids that include several regulatory motifs, normally blocking kinase activity.

In a previous study, we demonstrated that the transforming gene derived from G1III6 PBL was located on chromosome 7 by Southern blotting analysis of DNA from 12 different rodent-human somatic cell hybrids containing unique complements of the human chromosome, using 9F as the probe.[23] 9F is the subfragment of the genomic EMBL 3 clone 9-1 hybridizing to the kinase domain of the B-*raf* cDNA. Therefore, the B-*raf* gene is considered to be located on chromosome 7. In this paper we confirmed this fact by Southern blotting analysis of DNA from rodent-human hybrid cells, using the B-*raf* cDNA probe. While this paper was in preparation, Sithanandam *et al.*[36] reported that the B-*raf* gene was localized to human chromosome region 7q33-36 by Southern blot analysis of its segregation in a panel of rodent-human hybrids, using a B-*raf* cDNA clone and chromosomal *in situ* hybridization.

Northern blot analysis, on hybridization with probe C of the cloned cDNA, revealed two activated B-*raf* transcripts of 1.8 and 2.3 kb in the transformants. The same two transcripts were also shown to be hybridized with probes A and D (data not shown). We have not yet acquired full-length cDNA and have not found a variant cDNA clone with the inclusion of an additional exon or a partial deletion. Therefore, we do not know the identity of the mRNA to which the acquired cDNA corresponds. Alternative splicing or an alternative transcription start site may play a role in the generation of the divergent mRNA. Otherwise, another DNA rearrangement might occur during the transfection process. However, this hypothesis is not likely, since hybridization of transformant DNAs (S378A, S402, S424 and S448) with the cloned cDNA probe C (Fig. 1) revealed one DNA band, suggesting only one transforming DNA sequence in the transformants (data not shown).

In normal mouse tissues, 10 and 13 kb B-*raf* transcripts, which were most prominent in cerebrum, were detected as well as alternative-sized transcripts of 4.5 and 2.6 kb in testis, placenta and fetal membrane.[37] Thus B-*raf* expression is specific to each cell type in contrast to c-*raf*-1, which is expressed in many tissues and cell lines at fairly constant levels.[37] We found that human leukemia cell lines displayed transcripts of 4.0 and 2.6 kb, while other cell lines from brain tumors and a neuroblastoma did not (data not shown). B-*raf* might play an important role in the leukemogenesis of hematopoietic cells. Further studies are in progress to clarify the function of B-*raf* in tumorigenesis.

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